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Extensive Electrophoretic Variation in the Apogamous Fern Species, *Dryopteris nipponensis* (Dryopteridaceae)

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To determine genetic variation in the apogamous fern species *Dryopteris nipponensis*, allozyme analysis was carried out. Among the 196 individuals of *D. nipponensis* examined, 45 electrophoretically distinguishable variants were detected. This is the largest number of electrophoretic variants found in one apogamous fern species so far examined. Only one variant was characterized by a unique allele. All of the other variants were distinguished by the combinations of the limited number of alleles that were shared by some variants. The evidence strongly suggests that this apogamous species is of recurrent origin. The possibility of homoeologous chromosome pairing was also evaluated. Theoretically, genotypes of twelve of 45 variants can be generated from those of other variants via homoeologous chromosome pairing.

Key words: allozyme, apogamous species, clonal diversity, *Dryopteris nipponensis*, homoeologous chromosome pairing

Apogamy in ferns is a form of asexual reproduction in which unreduced spores are formed and the resultant gametophytes produce sporophytes without fertilization. In general, individuals produced by apogamy are clones of the parent. It is therefore expected that only minor variation will be seen in apogamous species. However, numerous apogamous species show great morphological variation, and often form species complexes that are taxonomically problematical. Allozyme studies also have shown genetic variation in *Asplenium unilaterale* Lam. *sensu lato* (Watano & Iwatsuki 1988), *Pteris cretica* L. (Suzuki & Iwatsuki 1990), *Dryopteris bissetiana* (Baker) C. Chr. (Lin *et al.* 1995), *Diplazium doederleinii* (Luerss.) Makino (Takamiya *et al.* 2001), and so on, while *Dryopteris yakusilvicola* Kurata (Darnaedi *et al.* 1990) and *D. remota* (A. Braun) Druce (Schneller & Holderegger 1994) showed no genetic variation. Those observations suggest that many apogamous species are not of single origin, but of recurrent origin. In addition, there may be other mechanisms by which genetic variation in apogamous species arises, such as homoeologous chromosome pairing (Klekowski 1973, Klekowski & Hickok 1974, Chapman *et al.* 1979, Masuyama 1984).

The *Dryopteris erythrosora* group (Miyamoto & Nakamura 1988, Dryopteridaceae) contains a number of apogamous species. Among about twenty taxa of this complex in Japan, only three species

(*D. caudipinna* Nakai, *D. koidzumiana* Tagawa, and *D. kinkiensis* Koidz. ex Tagawa) are sexual, while all of the other taxa are known to be apogamous (Iwatsuki 1992, Takamiya 1996). Most of those apogamous taxa are triploids. Morphologically, intermediate forms can be identified between various combinations of two taxa. With regard to the origin of apogamous fern species, hybrid origin has been documented through hybridization experiments and through studies of wild plants (Manton 1950, Walker 1962, Suzuki & Iwatsuki 1990, Darnaedi *et al.* 1990), as well as autopolyploid origin (Gastony 1988, Gastony & Windham 1989). It seems that complex speciation events involving hybridization and the acquisition of apogamous reproduction have occurred in the *Dryopteris erythrosora* group. *Dryopteris nipponensis* Koidz., one taxon of this group, is also a triploid apogamous species (Kurita 1966, Hirabayashi 1967, Ishikawa unpublished). It occurs mainly on Honshu, Shikoku, and Kyushyu in Japan, and rarely in China and South Korea (Oh 1978, Kurata & Nakaike 1985, Iwatsuki 1992, Flora of Zhejiang Editorial Committee 1993). *Dryopteris nipponensis* shows extensive morphological variability and is treated as a variety of *D. erythrosora* (D.C.Eaton) Kuntze by some authors (Ohwi 1957, Kurata & Nakaike 1985, Nakaike 1992), or as a distinct species by others (Ito 1939, Tagawa 1959, Iwatsuki 1992). Mitsuta & Nagamasu (1985) reported that *D. nipponensis* exhibits intermediate characters between *D. koidzumiana* and *D. formosana* (H.Christ) C.Chr. and is possibly of hybrid origin.

In this study, 188 individuals of *D. nipponensis* were collected from 19 localities covering the distribution area of the species in Japan, and eight from one locality in South Korea. Allozyme analysis was carried out to determine the genetic variation in this apogamous species. Below we discuss the causes of the polymorphism we observed.

Materials and Methods

Dryopteris nipponensis bears some resemblances to *D. erythrosora, D. caudipinna, D. koidzumiana,* and *D. formosana*. The following characters have been used to distinguish *D. nipponensis* (Iwatuski 1992). The basiscopic pinnules next to the rachis on the lowest pinnae are shorter or slightly longer than the adjacent pinnules. The basiscopic pinnules next to the rachis on the lowest pinnae are parted. The young fronds and indusia are not reddish.

Samples of *Dryopteris nipponensis* were collected from the localities given in Table 1. Approximately 200 stocks were planted in the field at the Faculty of Science, Chiba University, and used for allozyme analysis. Voucher specimens are deposited in the herbarium of the Natural History Museum and Institute, Chiba, Japan (CBM).

Fresh leaves were taken from living plants and analyzed electrophoretically. One hundred mg of leaf material was ground in 1 ml of extraction buffer composed of 0.1 M Tris-HCl pH7.5, 10 mM $MgCl_2$, 0.5 % Sodium metabisulfite, 0.5 % Sodium ascorbate, 8 % PVP (SIGMA, St. Louis, MO, USA), and 0.5 % 2-mercaptoethanol. The resulting slurry was centrifuged at 10000 rpm for 10 min, and the supernatant was used for electrophoresis.

Enzyme electrophoresis was conducted for the following seven enzymes: asparate aminotransferase (AAT/GOT), leucine aminopeptidase (LAP), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), 6-phsphogluconate dehydrogenase (6PGD), phosphoglucose isomerase (PGI), and triosephosphate isomerase (TPI). AAT and LAP were examined by the polyacrylamide gel system of Shiraishi (1988). IDH, MDH, and 6PGD were resolved on 11.5% starch gel and the histidine citrate buffer system of Cardy *et al.* (1981). PGI and TPI were electrophosed on 11.5% starch gel and buffer system 8 of Soltis *et al.* (1983). Staining schedules followed the methods of Shiraishi (1988) for AAT and LAP, and the methods of Soltis *et al.*

TABLE 1. Localities and voucher specimens of *Dryopteris nipponensis* used in this study

Symbol	Locality	No. of plants	Voucher
A	Asahi-mura, Niigata Pref.	2	HI95091201, HI95091208
B	Arakawa-cho, Niigata Pref.	1	HI95091209
C	Shibata, Niigata Pref.	1	HI95091215
D	Yahiko-mura, Niigata Pref.	1	HI95091319
E	Nishiyama-cho, Niigata Pref.	1	HI95091320
F	Suzu, Ishikawa Pref.	29	HI95093001, HI95093010, HI95093013, HI95093014, HI95093017
G	Anamizu-cho, Ishikawa Pref.	27	HI95093030, HI95093034, HI95093035, HI95093049, HI95093051
H	Kashima-cho, Ishikawa Pref.	11	HI95092901, HI95092904, HI95092907, HI95092908, HI95092909
I	Oamishirasato-cho, Chiba Pref.	4	HI94040209, HI94040227, HI94040229, HI94040230
J	Ichinomiya-cho, Chiba Pref.	1	HI94061807
K	Huttsu, Chiba Pref.	3	HI94030639, HI94030641, HI94030644
L	Amatsukominato-cho, Chiba Pref.	42	HI94022701, HI94041209, HI94041211, HI94041212, HI94041213
M	Oshima-cho, Tokyo Pref.	1	HI94060120
N	Eigenji-cho, Shiga Pref.	2	HI94080302, HI94080303
O	Owase, Mie Pref.	16	HI95040504, HI95040511, HI95040512, HI95040517, HI95040521
P	Nishinoshima-cho, Shimane Pref.	2	HI95040211, HI95040212
Q	Izuhara-cho, Nagasaki Pref.	9	HI95033005, HI95033008, HI95033013, HI95033033, HI95033034
R	Tarumizu, Kagoshima Pref.	8	HI95032701, HI95032704, HI95032801, HI95032802, HI95032806
S	Kamiyaku-cho, Kagoshima Pref.	27	HI94062504, HI94062513, HI94062519, HI94062606, HI94062612
T	Cheju Island, South Korea	8	HI95042201, HI95042308, HI95042319, HI95042325, HI95042331

(1983) for the other enzymes.

When multiple banding zones were observed, presumed loci were numbered sequentially with the most anodally migrating locus designated as 1. Allozymes at individual loci were lettered alphabetically, with the fastest migrating allozyme designated as a. The genotypes were interpreted based on the known subunit structure of the enzymes, and on the number of isozymes typical for each enzyme (Soltis *et al.* 1983, Weeden & Wendel 1989). As for the locus *Pgi-2*, the gene dosage was estimated based on the relative intensity of the stained bands. Genotypes of triploid plants were coded by three letters, such as *aab* and *bcc*. The genotypes of the other loci were coded only by the combination of alleles, such as a/b, and b/c, because we were not able to clearly identify the gene dosage for those loci.

Clonal diversity in 9 localities (F, G, H, L, O, Q, R, S, and T) was quantified by Simpson's diversity index D (Pielou 1969).

$$D=1-\sum\{[n_i(n_i-1)]/[N(N-1)]\}$$

where n_i is the number of individuals of variant i, and N is the number of collected individuals. The other localities were excluded in this analysis because the numbers of individuals collected were too small.

Results

Among the seven enzymes examined, the band patterns of MDH were too complex to be interpreted. AAT and TPI showed no genetic variation. Finally, we recorded the genotypes at four loci, *Lap, 6Pgd-2, Idh,* and *Pgi-2*. The bands at *Pgi-1* and *6Pgd-1* were not well resolved, and not scored.

At the locus *Lap*, six alleles were detected, and one to three alleles were found in each individual. Twelve different genotypes were distinguished by the combinations of alleles. At the loci *6Pgd-2, Idh,* and *Pgi-2*, three, two, and four alleles were detected, and three, two, and fifteen different genotypes were observed respectively. Representative band patterns of PGI-2 are shown in Fig. 1. Finally, by integrating the genotypes at four loci, 45 electrophoretically distinguishable variants (clones) were recognized in 196 individuals of *Dryopteris nipponensis*. They were named variant 1 to variant 45. The number of individuals and the genotype of each variant are shown in Table 2. The allele *Lap-a* was observed only in variant 1. All alleles except Lap-a were shared by more than two variants.

The number of individuals, number of variants, and Simpson's diversity index (D) in each locality are shown in Table 3 and Fig. 2.

Discussion

In this study, 45 electrophoretically distinguishable variants were detected. In previous studies on the genetic variation in apogamous ferns, 14 variants in *Dryopteris bissetiana* (Lin *et al.* 1995), six triploid and five diploid variants in *Pteris cretica* (Suzuki & Iwatsuki 1990), and one tetraploid and four triploid variants in *Diplazium doederleinii* (Takamiya *et al.* 2001) have been reported. In the asexually-reproducing gametophytic populations of *Vittaria appalachiana* Farrar & Mickel, ten variants were recognized (Farrar 1990). The number of variants in *Dryopteris nipponensis* is far larger than in those species.

Among the 45 variants, 23 variants (51%) were solitary, and 33 variants (73%) were found

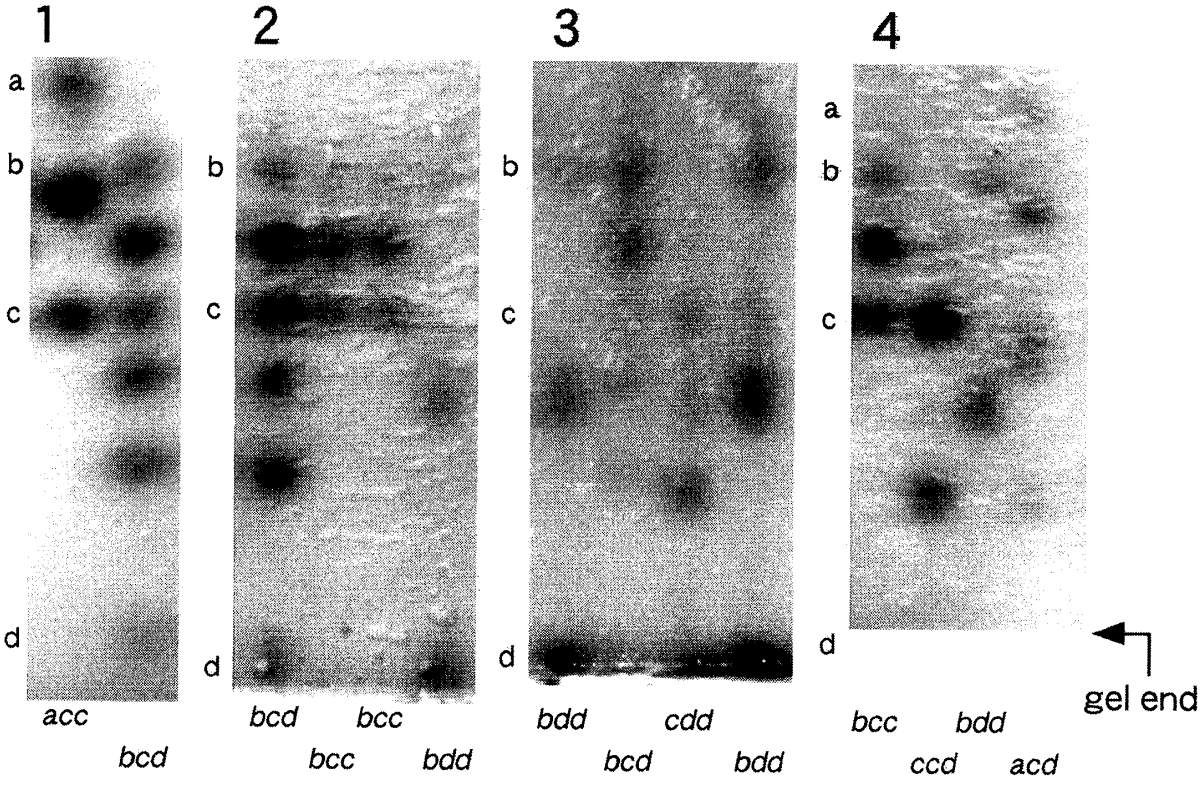

FIG. 1. Representative band patterns of PGI-2. The positions of homodimers are shown by the letters on the left side of the photographs. Homodimer dd is located out of the gel in photograph 4. The interpreted genotypes are shown below bands.

TABLE 2. Interpreted genotypes of 45 variants of *Dryopteris nipponensis* at four loci

Variant	No. of plants	No. of plants from locality	Lap**	6Pgd-2**	Idh**	Pgi-2
1	2	2T*	a/c	b/c	a	aac
2	19	3I, 1K, 14L, 1T	b	a/c	a	bbc
3	1	1R	b/c	-***	a	abc
4	1	1S	b/c	b/c	a	ccd
5	1	1Q	b/c	c	a/b	bcc
6	1	1S	b/d	a/c	a	bdd
7	2	2S	b/d	b/c	a	ccd
8	1	1I	bde	a/c	a	acc
9	1	1S	bde	b/c	a	ccd
10	1	1S	b/e	-	a	bdd
11	1	1J	b/e	a/c	a	aab
12	1	1L	b/e	a/c	a	ccd
13	1	1L	b/e	b/c	a	aab
14	1	1S	b/e	b/c	a	acd
15	17	14O, 3R	b/e	b/c	a	bbd
16	2	2S	b/e	b/c	a	bcd
17	3	1L, 2Q	b/f	a/c	a	abd
18	1	1L	b/f	a/c	a	bbd
19	9	9G	b/f	a/c	a	bcd
20	6	4L, 2O	b/f	b/c	a	bcd
21	2	1H, 1S	b/f	b/c	a	bdd
22	1	1P	c	a/c	a	bcd
23	1	1T	c	b/c	a	ccc
24	29	2A, 1E, 16F, 10H	c/e	a/c	a	aab
25	1	1L	c/e	a/c	a	abd
26	3	1R, 2S	c/e	a/c	a	bcc
27	1	1P	c/e	a/c	a	bcd
28	4	4S	c/e	a/c	a	cdd
29	2	2L	c/e	a/c	a	ddd
30	2	1G, 1L	c/e	b/c	a	aab
31	41	1B, 1C, 1D, 13F, 17G, 2L, 1M, 3Q, 2T	c/e	b/c	a	abc
32	1	1T	c/e	b/c	a	abd
33	1	1R	c/e	b/c	a	acc
34	10	1K, 5L, 1Q, 2R, 1S	c/e	b/c	a	bcd
35	2	2S	c/e	b/c	a	bdd
36	1	1T	c/f	-	-	bbd
37	3	3L	c/f	a/c	a	bcd
38	4	4S	c/f	a/c	a/b	cdd
39	1	1S	c/f	b/c	a	abc
40	4	1K, 3L	c/f	b/c	a	abd
41	5	3L, 2S	c/f	b/c	a	bdd
42	1	1S	c/f	b/c	a/b	bdd
43	1	1N	d/e	a/c	a	aab
44	1	1N	d/e	a/c	a	abc
45	2	2Q	d/f	b/c	a	abc

* Symbol of locality is shown in Table 1. 2T indicates 2 plants from locality T.

** Combinations of alleles are shown. See text.

*** No data.

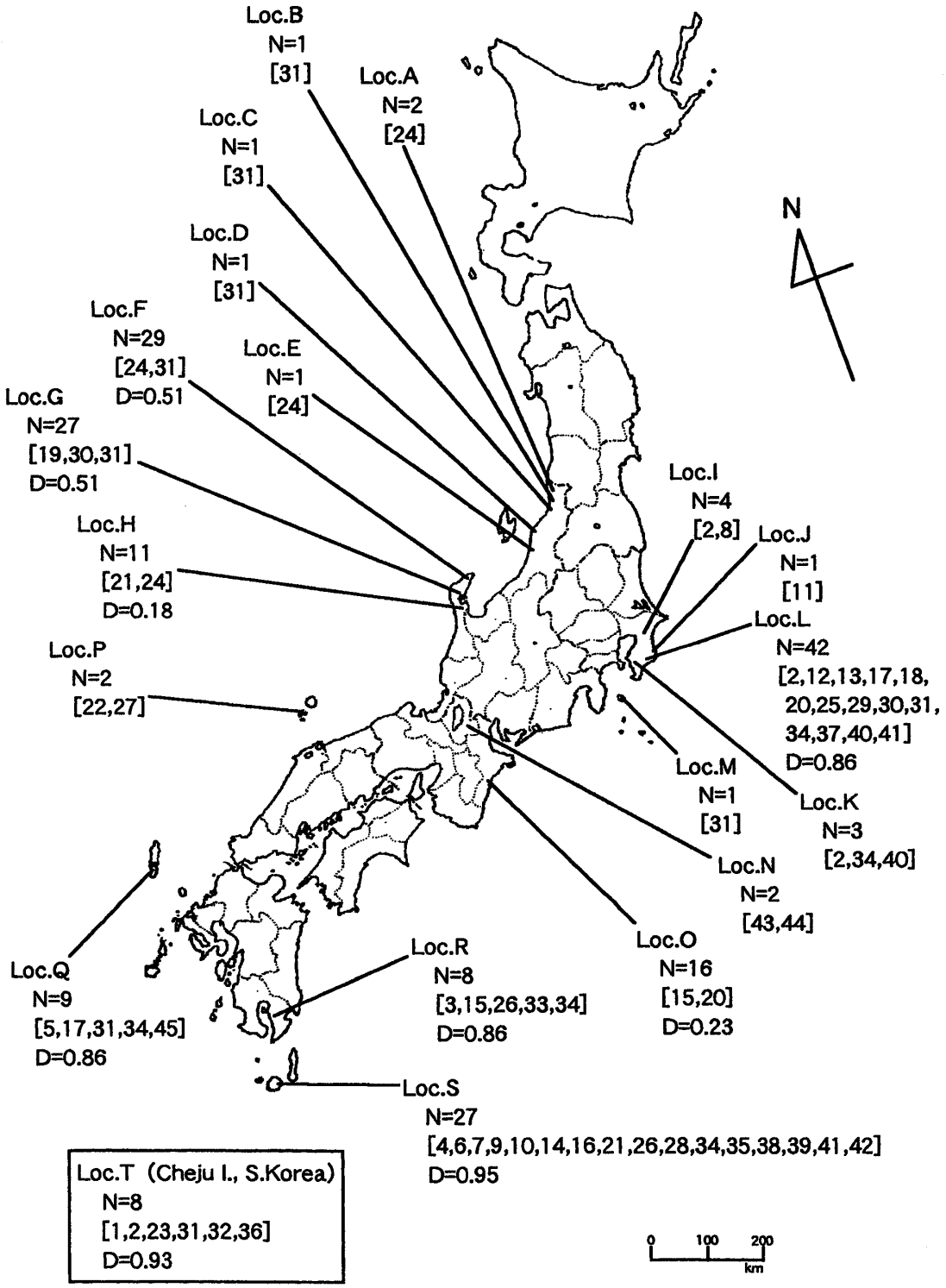

FIG. 2. Variants, number of plants, and diversity index in each locality. Symbols of localities are shown in Table 1. N: Number of plants. D: Simpson's diversity index. Variants found in each locality are shown in brackets.

TABLE 3. Number of plants, number of variants and clonal diversity in each locality

Locality	No. of plants	No. of variants	Variant	Diversity index
A*	2	1	24	-**
B	1	1	31	-
C	1	1	31	-
D	1	1	31	-
E	1	1	24	-
F	29	2	24, 31	0.51
G	2	3	19, 30, 31	0.51
H	1	2	21, 24	0.18
I	4	2	2, 8	-
J	1	1	11	-
K	3	3	2, 34, 40	-
L	42	14	2, 12, 13, 17, 18, 20, 25, 29, 30, 31, 34, 37, 40, 41	0.86
M	1	1	31	-
N	2	2	43, 44	-
O	16	2	15, 20	0.23
P	2	2	22, 27	-
Q	9	5	5, 17, 31, 34, 45	0.86
R	8	5	3, 15, 26, 33, 34	0.86
S	27	16	4, 6, 7, 9, 10, 14, 16, 21, 26, 28, 34, 35, 38, 39, 41, 42	0.95
T	8	6	1, 2, 23, 31, 32, 36	0.93

* Symbol of locality is shown in Table 1.

** Not calculated. See text.

in only one locality (Table 2), indicating that the clones of *D. nipponensis* have a highly restricted distribution pattern. A similar pattern of clone distribution is reported for many agamospermous and vegetatively reproducing clonal seed plants (Ellstrand & Roose 1987). Considering the highly restricted distribution pattern of each variant, 45 may be fewer than the actual number of existing clones of *D. nipponensis*. A larger scale sampling may reveal a greater number of variants.

The possible causes for genetic variation in apogamous species are mutation (Manton 1950), homoeologous chromosome pairing (Klekowski 1973, Klekowski & Hickok 1974, Chapman *et al.* 1979, Masuyama 1984), and recurrent origin (Walker 1962, Watano & Iwatuski 1988, Suzuki & Iwatsuki 1990, Lin et al. 1995).

In this study, only one unique allele was found at one locus of variant 1 (*Lap-a*). The rarity of unique alleles suggests that the contribution of mutation to genetic variation in *D. nipponensis* is not significant. All alleles except *Lap-a* were shared by more than two variants. As a result, most of the variants are distinguished by the combinations of a limited number of alleles, which implies that *D. nipponensis* originated multiple times independently

TABLE 4. Possible combinations of ancestor-descendant pairs of variants under assumed homoeologous chromosome pairing involving the locus Pgi-2

Variants	Genotypes
16 → 15	bcd → bbd
17 → 18*	abd → bbd
19 → 18	bcd → bbd
20 → 21	bcd → bdd
25 → 24	abd → aab
27 → 26	bcd → bcc
27 → 28	bcd → cdd
28 → 29	cdd → ddd
31 → 30*	abc → aab
32 → 30	abd → aab
31 → 33	abc → acc
32 → 35*	abd → bdd
34 → 35	bcd → bdd
40 → 41	abd → bdd
44 → 43	abc → aab

*Variants 18, 30 and 35 can be derived from two ancestral variants each.

from limited genetic resources.

The pattern of genetic variation in *D. nipponensis* does not preclude the possibility of homoeologous chromosome pairing. There has been no electrophoretic evidence for homoeologous chromosome pairing in ferns (Gastony & Gottlieb 1982, Gastony & Darrow 1983). However, there have been few electrophoretic studies that have verified the possibility of such a mechanism, and it is still a controversial topic. The sporogenesis pathway in the apogamous species of *Dryopteris* follows the Döpp-Manton scheme (Manton 1950, Kurita 1967, Hirabayashi 1974), and potentially homoeologous chromosome pairing can take place (Klekowski 1973). For example, variants 30 and 31 have the same genotype at *Lap, 6Pgd-2*, and *Idh*, but different genotypes at *Pgi-2, aab*, and *abc*. If homoeologous chromosome pairing and the resulting segregation in the progeny occur, the genotype *aab* of variant 30 can be generated from the *abc* of variant 31. Theoretically, genotypes of twelve of the 45 variants of *D. nipponensis* can be induced from other variants by segregation of the *Pgi-2* genotypes through homoeologous chromosome pairing (Table 4). To examine segregation of genotypes in progeny under experimental conditions, spores were collected from one sporophyte with the genotype *abc* at *Pgi-2* (variant 31, locality M), and gametophytes and offspring sporophytes were prepared. As a result, five of 284 progenies (250 gametophytes and 34 sporophytes) showed different genotypes (three *aac*, one *bbc*, and one *bcc*) from that of the parent, which can be explained by segregation through homoeologous chromosome pairing (Ishikawa *et al.* in press).

Although homoeologous chromosome pairing is one of the possible causes of genetic variation in *D. nipponensis*, the origins of all variants cannot be explained only by this mechanism. For example, variants with the genotype *abc* at *Pgi-2* are incapable of producing genotypes with allele *d*, such as *abd*, by homoeologous chromosome pairing. Consequently, it is almost certain that *D. nipponensis* is not of a single origin. Presumably, *D. nipponensis* has arisen many times independently.

Diversity index (Table 3, Fig. 2) is high in localities L, Q, R, S, and T (D ranges from 0.86 to 0.95), and low in localities F, G, H, and O (D ranges from 0.18 to 0.51). There are only two variants (24 and 31) in 35 individuals collected from six localities A, B, C, D, E, and F. It appears that clonal diversity is lower in the northern part of the distribution area of *D. nipponensis* than in other parts. This may reflect the history of geographical expansion of *D. nipponensis*. Additional analyses with standardized sampling methods should be carried out.

An apogamous species may arise from the results of hybridization between two sexual individuals, followed by the acquisition of apogamous reproduction through mutation (Darnaedi *et al.* 1990). Alternatively, hybridization between a sexual individual and an apogamous one can produce a new apogamous hybrid (Walker 1962, Watano &

Iwatsuki 1988, Suzuki & Iwatsuki 1990). It has been pointed out that *D. nipponensis* is morphologically intermediate between *D. koidzumiana* and *D. formosana*, and is of possible hybrid origin (Mitsuta & Nagamasu 1985). Since *D. nipponensis* is probably of recurrent origin, if both parents were sexual, apogamous reproduction should have evolved recurrently. This is not very likely to have occurred, although it cannot be ruled out absolutely. It seems more reasonable that one of the parents of *D. nipponensis* was an apogamous type. The available data show that *D. koidzumiana* is a diploid sexual species, and *D. formosana* is a triploid apogamous species (Takamiya 1996). Because *D. nipponensis* is a triploid, the origin of *D. nipponensis* can not be explained by simple hybridization between the known cytotypes of *D. koidzumiana* and *D. formosana*. To clarify the origin of *D. nipponensis* it is therefore necessary to carry out an extensive survey of cytotypes in related species, including the putative ancestors, and to investigate phylogenetic relationships among them by using genetic markers such as allozymes, plastid DNAs, and nuclear DNAs.

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